

# Developmental changes in estrogen-sensitive neurons in the forebrain of the zebra finch

(brain/gender difference/antibody)

MANFRED GAHR AND MASAKAZU KONISHI

Division of Biology, 216-76, California Institute of Technology, Pasadena, CA 91125

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**ABSTRACT** The brain areas for the control of song are sexually dimorphic in the zebra finch (*Poephila guttata*). Implantation of estrogen in young females within the first 40 days after hatching masculinizes their brain song areas. Monoclonal antibody (H222Spy) against the estrogen receptor was used for the localization of estrogen-target cells in the brain. The nucleus hyperstriatum ventrale, pars caudale, was the only song control area that contained cells with estrogen-receptor sites. The number of these cells varied during ontogeny and declined sharply after day 40. No other song areas contained estrogen-target cells despite their ability to undergo masculine differentiation under the influence of estrogen. Therefore, the action of estrogen on these nuclei must be indirect.

A set of brain nuclei controls song production in songbirds (1). In the zebra finch (*Poephila guttata*) the song control areas are highly sexually dimorphic (2). Female zebra finches do not sing and their song nuclei are much smaller than those of males. The sex differences arise from higher rates of neuronal atrophy and death in the female song nuclei and from an increase in cell size and number in the male (3–5). Administration of estrogen to a juvenile female finch induces male-like differentiation in her song nuclei (6, 7). This estrogen-mediated differentiation is only inducible in a limited phase of ontogeny—from the first day of hatching to about day 40 (8). The inductive action of estrogens on cells is thought to be mediated by special intracellular receptor molecules (9). Such cells can be identified and localized by immunocytochemical methods using monoclonal antibodies against the estrogen receptor (10). The present study investigates developmental changes in the number and distribution of estrogen-binding cells in and near the forebrain song nuclei [ventral nucleus of hyperstriatum caudal section (HVc) and robust nucleus of archistriatum (RA)] in the female zebra finch.

## MATERIALS AND METHODS

**Comparison Between Estrogen Autoradiography and Estrogen-Receptor Immunocytochemistry.** Because the antibody (H222Spy) used in this study was originally developed from human breast cancer cells (11, 12), its ability to cross-react with the finch estrogen receptor was tested by comparison with estrogen autoradiography. Two adult male zebra finches were castrated, and 24 hr later each bird was injected intramuscularly with 15  $\mu$ g of 17 $\beta$ -[<sup>3</sup>H]estradiol per g of body weight [New England Nuclear no. NET517; specific activity = 35 Ci/mmol (1 Ci = 37 GBq); 10  $\mu$ Ci per g of body weight] dissolved in 70% ethanol. The birds were killed 90 min after the injection and the brains were removed and frozen over liquid nitrogen. Brains were cut into 10- $\mu$ m sagittal sections with a cryostat and the sections were mounted onto photoemulsion (Kodak NTB 3)-coated slides. The slides were

stored in lightproof boxes at –70°C for 6 months. Sections were fixed in buffered formaldehyde, developed with buffered Kodak D19, fixed with buffered Kodak Unifix, and stained with the estrogen-receptor antibody H222Spy after the methods described elsewhere (10). A cell was considered to be labeled if it had five times more silver grains over its soma than adjacent cell-sized areas of neuropil. For quantitative comparison of antibody and <sup>3</sup>H labeling, five sections from each area were examined.

The distribution of antibody-labeled neurons was very similar to that of <sup>3</sup>H-labeled neurons throughout the brain. However, the number of antibody-labeled cells was in all areas greater than that of <sup>3</sup>H-labeled cells. Double-labeled cells (Fig. 1) were found in all areas that contained <sup>3</sup>H-labeled neurons. The ratio of double-labeled neurons to the total number of either <sup>3</sup>H- or antibody-labeled neurons varied between different areas, including the preoptic area, the hypothalamus, the occipitomesencephalic fiber tract, the areas along the ventral ventricle, and in and around the HVc. Seventy-two percent (range, 48–89%) of <sup>3</sup>H-labeled cells were double-labeled, with the highest number of double-labeled neurons around the HVc (89%) and in the occipitomesencephalic fiber tract (78%). On the other hand, 59% (range, 27–72%) of antibody-labeled cells were double-labeled, with the highest percentage in the hypothalamus (72%) and around the HVc (71%). The autoradiographic procedures appear to affect the proportion of antibody-labeled cells. Thus, the number of antibody-labeled neurons of sections stained directly after cutting the brain was 20–30% higher than that of comparable sections stained after the autoradiographic procedures.

These discrepancies between the two methods may be due to the following causes: loss of antigenic sites by the autoradiographic procedures, incomplete saturation of estrogen-receptor sites with [<sup>3</sup>H]estrogen, estimation of <sup>3</sup>H-labeled cells with the five times background criterion, and thickness of the sections. Nevertheless, the good agreement between autoradiography and antibody labeling suggests that H222Spy recognizes most estrogen-receptor types. Also, double labeling of neurons with the antibody and 17 $\beta$ -[<sup>3</sup>H]estradiol shows that antibody-labeled cells contain functionally active estrogen receptors. Siegel *et al.* (13) showed that androgen and estrogen receptors of the zebra finch brain are similar to those of other vertebrate brains. Furthermore, recent studies show that identical amino acid sequences occur in human and avian estrogen-receptor molecules (14, 15).

**Immunocytochemical Procedures.** Birds of known ages were obtained from our breeding stock. We compared the number of antibody-labeled cells in different age groups, including posthatching day 20, 30, 40, 45, and 50 and adult. A sample of five birds was used for each age group. Birds were overdosed with equithesin and perfused intracardially first with 0.9% saline, then with 4% ice-cold phosphate-

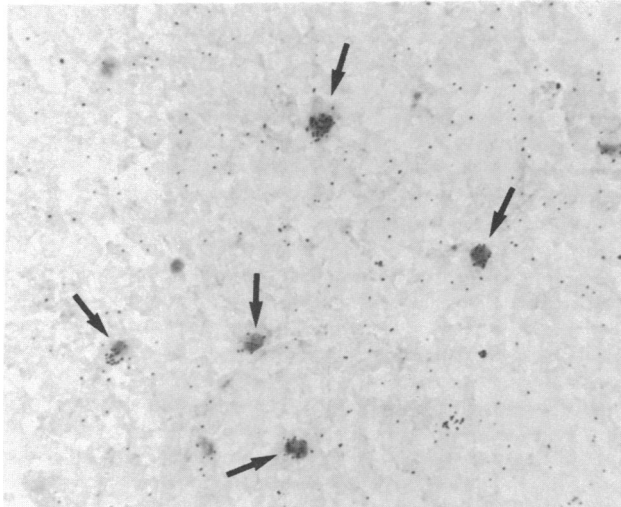


FIG. 1. Comparison between autoradiography and immunocytochemistry. Double-labeled hypothalamic neurons, shown by arrows, contain immunocytochemical reaction products and silver grains over their somata. There is a good agreement between the two methods. ( $\times 1360$ .)

buffered formaldehyde in 0.9% saline, and finally with 10% ice-cold phosphate-buffered sucrose in 0.9% saline. Brains were removed and immersed in 30% buffered sucrose before they were cut on a freezing microtome. Brains were cut into 30- $\mu$ m parasagittal sections and collected in phosphate-buffered saline (PBS); sections were treated with 0.1% buffered Triton X-100 for 30 min. After sections were washed in PBS, they were immersed in 3% normal rabbit serum for 30 min before incubation in the primary antibody H222Spy (Abbott) (10  $\mu$ g/ml in 0.1 M PBS) for 12 hr. The sections were washed again in PBS and then incubated in biotinylated secondary antibody (14  $\mu$ l/ml in 0.1 M PBS) (Vector Laboratories, Burlingame, CA; no. 4004) for 1.5 hr. After the sections were washed, they were incubated in the Vectastain ABC reagent (Vector Laboratories; no. 4004) for 1 hr. The sections were then incubated for 7–10 min in the chromagen medium containing diaminobenzidine (0.2 mg/ml) and hydrogen peroxide (0.003%) and osmicated for visualization of antigen–antibody complex. Finally, sections were placed on gelatin-coated slides and covered with coverslips.

For control of the specificity of the staining, sample sections were stained by the same procedures except that the primary antibody was replaced by normal rat IgG, or the first, second, or third antibody was omitted, or hydrogen peroxide was omitted. In all these cases, no labeled cells were found.

The loci and numbers of labeled cells were determined under a Zeiss microscope with the aid of a camera lucida. Relevant sections were drawn and the position of each labeled neuron was marked. These sections were then counterstained with thionin and the borders of song nuclei were drawn. The drawings of antibody-labeled and thionin-stained sections were superimposed on each other and the number of antibody-labeled neurons per area was counted. The size of the drawn areas was measured by a computer-aided planimetric method. The volume of the tissue within the areas was derived from the above measurement. Neuron density was estimated on counterstained sections by counting neurons under high magnification with the aid of an ocular grid. The total neuron number per area was then derived from neuron density and the volume of the tissue. The numbers of antibody-labeled neurons were compared between age groups by a  $\chi^2$  test.

Because the proportion of antibody-labeled neurons to all neurons was small, minor variation in cell count in an area

caused the proportion to change too drastically for it to be useful as an index for comparison. Also, as it is not known whether it is the total number or the proportion of target neurons that is significant for hormone action, we present the number of antibody-labeled neurons and the estimated total of all neurons.

## RESULTS

The distribution of antibody-labeled neurons in the brain of juvenile zebra finches was similar to the adult pattern described elsewhere (10). The following areas contained antibody-labeled neurons: the preoptic area including nucleus preopticus anterior and nucleus preopticus medialis; hypothalamic nuclei including nucleus periventricularis magnocellularis; the infundibulum and nucleus medialis hypothalami posterioris; limbic structures such as lateral septum and nucleus taenie; mesencephalic areas such as nucleus intercollicularis and the central gray; and the nucleus tractus solitarius in the rhombencephalon. As an example, Fig. 2 shows antibody-labeled neurons in the hypothalamic areas of a 50-day-old female zebra finch.

Among the song control nuclei of juvenile female zebra finches, the HVC is the only nucleus that contained antibody-labeled neurons (Fig. 3). A large number of antibody-labeled neurons were found in the mediocaudal telencephalon that includes the HVC. Many antibody-labeled neurons occurred near the border between the HVC and the caudate neostriatum. It was sometimes difficult to decide by cytoarchitectonic criteria whether such neurons belonged to the HVC. Therefore, for ease of comparison between different age groups, we divided the mediocaudal telencephalon into two areas: HVC itself and a 100- $\mu$ m zone surrounding the HVC. When a neuron could not be placed inside or outside the HVC, it was regarded as belonging to the surrounding area.

Antibody-labeled neurons were not homogeneously distributed in the HVC but were found predominantly in the mediocaudal part of the nucleus. Thus, the number of labeled neurons in certain parts was much higher than the average number of labeled neurons for the whole nucleus. The highest number of labeled neurons was found in 30-day-old birds (Table 1) (Fig. 3).

The median number of antibody-labeled neurons in the HVC was not different between 20-, 30-, and 40-day-old

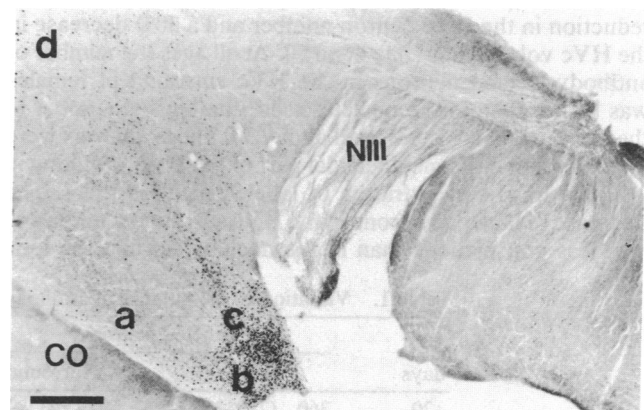


FIG. 2. Antibody-labeled neurons in the hypothalamus of a 50-day-old female zebra finch. In this sagittal section a band of antibody-labeled neurons extends from the preoptic area along the optic chiasm (CO) (a) into the infundibulum (b). Another band of labeled neurons stretches from the infundibulum continuously into the medial nucleus of the posterior hypothalamus (c). Some labeled neurons occur in the anterior hypothalamus. "d" indicates labeling in the nucleus periventricularis magnocellularis. NIII is the oculomotor nerve. (Bar = 50  $\mu$ m.)

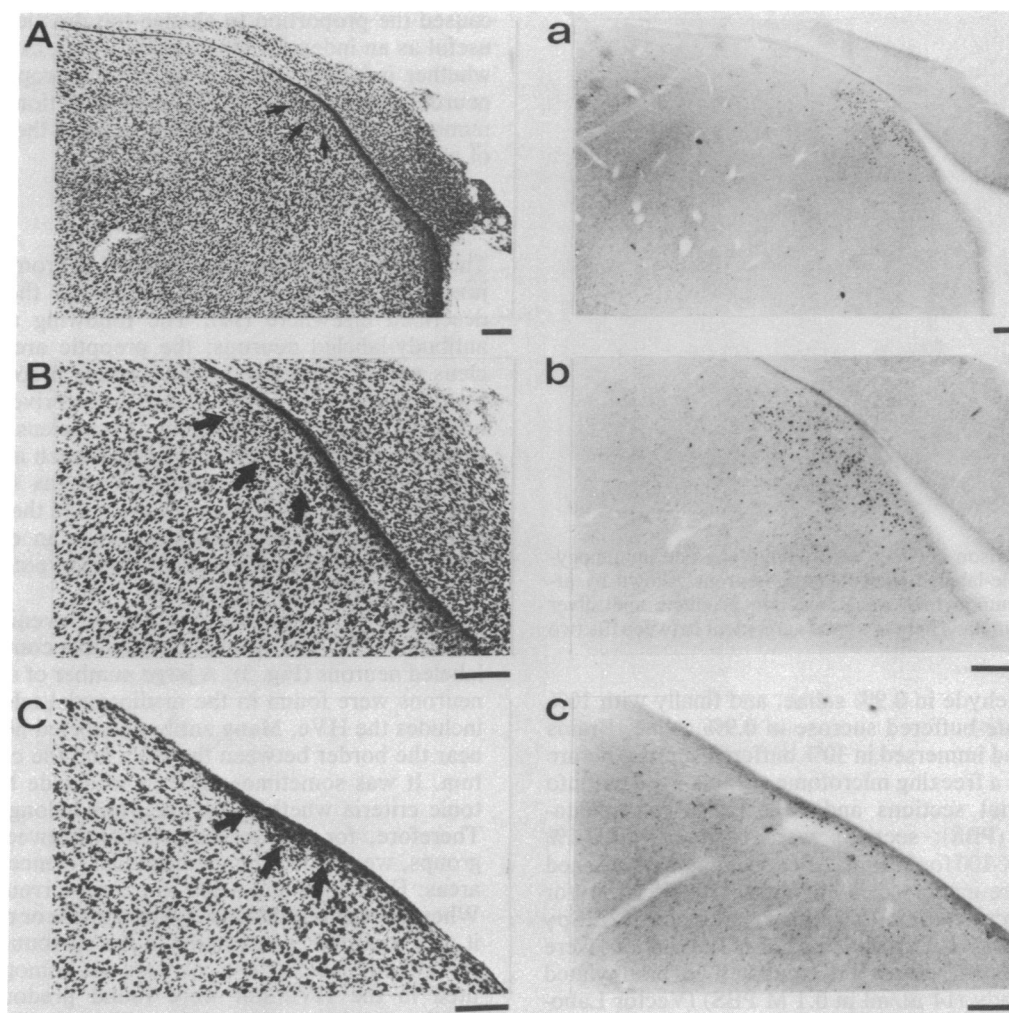


FIG. 3. Antibody-labeled neurons in and around the HVc. (A and a) Low-power photomicrographs of the HVc of a 30-day-old female showing parasagittal sections stained with thionin (A) or with the estrogen-receptor antibody (a). (B and b) High-power versions of A and a, respectively. (C and c) High-power photomicrographs of the HVc of an adult female showing parasagittal sections stained with thionin (C) or with the antibody (c). Estrogen receptor-containing neurons are darkly labeled. The HVc is indicated by arrows in A, B, and C. (Bars = 50  $\mu$ m.)

females but declined significantly between day 40 and day 50 ( $P < 0.01$ ,  $\chi^2$  test). This decrease coincided with a 30% reduction in the HVc neuron number and a 30% decrease in the HVc volume from day 40 to 50. At all ages the number of antibody-labeled neurons in the HVc surround of females was higher than in the nucleus itself; the highest number in the surround was again found at day 30. These neurons were located around the mediocaudal part of the HVc, resulting in a higher local concentration of labeled neurons there. The decrease of labeled neurons in the HVc surround after day 40 was less conspicuous than in the nucleus, so that the total

numbers of labeled neurons in this area at days 40, 45, and 50 were still significantly ( $P < 0.01$ ) larger than in that of the adult female (Fig. 3).

## DISCUSSION

Siegel *et al.* (13), using DNA-cellulose chromatography, showed that the forebrain of male and female zebra finches contains androgen and estrogen receptors. Walters and Harding (16), using microdissected tissue, assayed estrogen receptors in four of the forebrain song nuclei in castrated

Table 1. Variation in the number of antibody-labeled neurons during development

Age, days	Labeled neurons*		Total neuron number in HVc*		HVc volume, mm <sup>3</sup>
	HVc	Surround			
20	360 (279–396)	592 (432–903)	—	—	—
30	480 (282–910)	550 (474–960)	24,000 (23,000–27,000)	0.067	0.067
40	342 (200–860)	610 (459–804)	19,600 (17,000–25,000)	0.051	0.051
45	128 (84–183)	432 (273–600)	—	—	—
50	125 (80–185)	360 (300–390)	12,500 (11,000–14,000)	0.033	0.033
Adult	9 (3–36)	135 (108–270)	7,300 (7,900–6,300)	0.021	0.021

The results are from five animals in each age class. The number of antibody-labeled neurons in the HVc decreases between day 40 and day 50. The number of labeled neurons in the HVc surround declines steadily after day 40. A dash indicates no measurement was made.

\*Median number, with range in parentheses.

male zebra finches. However, these techniques do not show the exact distribution of receptor-containing neurons. Furthermore, tissue samples from some of the song nuclei may be contaminated by estrogen-absorbing neurons that occur near the nuclei (17). Nordeen *et al.* (18), using the methods of estrogen autoradiography, found few  $^3\text{H}$ -labeled cells in the forebrain song nuclei of juvenile female and adult male zebra finches. A small number of lightly labeled cells can cause errors in identification and in statistical estimation. The method used in the present study overcomes some of the difficulties of biochemical and autoradiographic methods. For example, the antibody stained neurons uniformly so that all labeled neurons could be identified. This attribute and the good agreement between the autoradiographical and immunocytochemical results justify the assumption that the failure of antibody labeling indicates the absence of estrogen-absorbing neurons or a subthreshold receptor concentration.

The results presented above show that antibody-labeled neurons occur at all ages in and around the HVC. However, the number of estrogen-target neurons varies with age during the first 40 days after hatching. There is a sharp decrease in the number of estrogen-target neurons after 40 days of age, when a faster rate of neuronal loss takes place in the HVC. Also, the decline appears to coincide with the stage in which estrogen begins to lose its masculinizing effects on neurons of female HVC and RA (8). Estrogen-induced masculinization of female HVC may initially involve the prevention of death among receptor-containing neurons. These neurons may then maintain the survival and growth of other HVC neurons lacking the receptor. When these neurons die or undergo atrophy, estrogen has fewer and smaller targets. Although this scenario assumes cell death as a cause of the reduction in labeled neurons, other causes such as intracellular reduction of the receptor level and the loss or masking of the immunoreactive epitope of the receptor cannot be excluded.

Neurons of all forebrain song nuclei undergo masculine differentiation in response to estrogen treatment (6, 7). Yet only the HVC region contains estrogen-absorbing neurons in juvenile female zebra finches. These findings suggest indirect mechanisms for estrogen action in forebrain nuclei other than the HVC. Estrogen-target cells elsewhere may produce a substance that acts on neurons of other nuclei. Also, the cascading effects of differentiation in the HVC may spread to other nuclei by direct and indirect connections. For example, when the afferents to the RA of a 15-day-old male finch are cut, the nucleus on the operated side undergoes atrophy, whereas the one on the other side grows normally (19). A well-established case of indirect action of hormone on neurons is found in the androgen-dependent neuronal differen-

tiation of the bulbocavernosus nucleus of the rat spinal cord. The differentiation of these neurons appears to be maintained indirectly by the action of androgen on the muscles they innervate (20, 21). Thus, within the nervous system the primary site of hormone action can be remote from the site of cellular differentiation.

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